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14. ABSTRACT

Cyclin E is a positive regulator of the G1 to S phase transition of the cell cycle. In complex with CDK2 it is responsible for cells passing the restriction point, committing the cell to a round of DNA replication. Previously this laboratory found that cyclin E is overexpressed and present in lower molecular weight (LMW) isoforms in breast cancer cells and tumor tissues compared to normal cells and tissues. To investigate the role of the LMW forms of cyclin E in tumorigenesis we have developed a model system of non-tumorigenic breast cells overexpressing the individual isoforms of cyclin E. Using this model system we have determined that the LMW forms of cyclin E are associated with increased kinase activity that results in cell cycle deregulation, chromosomal instability and nare.

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Table of Contents

Page

Introduction:

The frequent appearance of the low molecular weight (LMW) forms of cyclin E and their correlation with poor prognosis in breast cancer patients indicate that the LMW forms may be oncogenic, playing specific roles in the development of malignancies. The purpose of this application was to determine the role of the LMW forms of cyclin E in the transformation of normal cells in to neoplastic cells. The outcome of this research will be significant in that it will delineate a novel mode of deregulation of cyclin E and possibly identify a new oncogene involved in breast cancer tumorigenesis therefore providing the rationale for novel drug design.

Body:

To determine the role of the low molecular weight (LMW) isoforms of cyclin E in tumorigenesis, we generated a model system of immortalized, non tumorigenic mammary epithelial (76NE6) cells overexpressing the full-length (EL) and LMW (T1) isoforms of cyclin E. We characterized the clones overexpressing the individual isoforms and compare these cells to the untransfected and vector alone transfected cells. We have shown that the LMW isoforms of cyclin E have increased kinase activity compared to the full length cyclin E. This activity translates in to an increased percentage of cells in S phase of the cell cycle. However, we could not conclude that this was due to increased proliferation in these cells due to inconsistent growth curve data. Therefore, we initially aimed to study the duration and timing of entry in to each phase of the cell cycle for the 76NE6 clones overexpressing the EL compared to T1 isoform of cyclin E. To this end, we synchronized the cells using growth factor depletion as described in materials and methods. Because the parental, 76NE6, cells are non-tumorigenic and have not lost their requirements for growth factors, removal of growth factors should result in the cells exiting the cell cycle in to the G0 phase until they receive a signal that there are growth factors available again for re-entry in to the cell cycle. Figure 1A shows a representative graph of the percentage of cells in G0/G1 phase of the cell cycle after release from 72 hours of growth factor deprivation. The empty vector and EL overexpressing cells synchronized efficiently upon removal of growth factors, with 74% and 67% of the cells entering G0/G1 phase respectively by the time the cells were released from arrest (time=0 hr). These cells then progressed synchronously through the phases of the cell cycle. On the other hand, only 50% of the T1 overexpressing cells were in G0/G1 phase after 72 hours of growth factor deprivation. After serum and growth factors were added back to the cells, the T1 cells did not appear to cycle through the cell cycle phases. Despite several attempts at synchronizing the cells, the T1 overexpressing cells were not becoming synchronous in their cycling. Therefore, we could not determine anything about the timing of phase entry.

The T1 overexpressing 76NE6 cells have a deregulated cell cycle, presumably due to defects in the G1/S checkpoint as a result of LMW cyclin E overexpression. Therefore, we hypothesized that cells overexpressing the LMW isoform (T1) of cyclin E do not exit the cell cycle under growth factor deprived conditions. To determine how the T1 overexpressing cells are reacting to removal of growth factors compared to the EL or empty vector overexpressing cells, we collected cells every 12 hours during the 120 hours of growth factor removal and analyzed their DNA content by flow cytometry to determine their position in the cell cycle. Figure 1B shows the percentage of cells from each clone in G0/G1 phase of the cell cycle over the 120hr period of growth factor deprivation. The results show that within 50 hours of growth factor deprivation, 100% of the 76NE6 cells overexpressing the empty vector have arrested their cell cycle at either G0 or G1 phase of the cell cycle. These 2 cell cycle phases fraction have the same DNA content and therefore they can not be differentiated by PI staining and flow cytometric analysis, but can be differentiated with Ki67 staining (Figure 2). By 120 hours, 65% of the EL overexpressing cells were arrested in G0/G1 phase. Although not all EL cells arrested in G0/G1, the population that was arrested remained in the G0/G1 phase of the cell cycle. In comparison, at 120 hours of growth factor deprivation, less than 50% of the T1 cells were in the G0/G1 phase of the cell cycle. These experiments show that the empty vector expressing cells quickly exit the cell cycle (all of the cells are in the G0/G1 DNA content peak), the EL overexpressing cells are attempting to arrest,

despite overexpression of cyclin E, but the T1 overexpressing cells show no signs of synchrony over the 120 hour period.

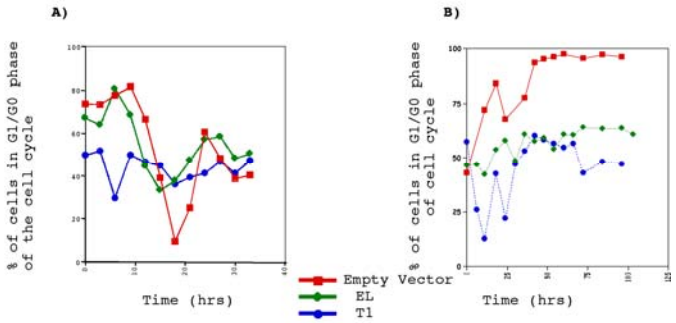

Figure 1: 76NE6 cells overexpressing T1 do not exit the cell cycle in response to growth factor deprivation. 76NE6 clones were subjected to growth factor deprivation. Cells were collected every A) 3 hours after re-addition of serum and subjected to FACS analysis, or B) every 12 hours during 120 hours of serum deprivation and subjected to flow cytometric analysis using propidium iodide staining of the cells and graphed as the percentage of cells in G0/G1 phase of the cell cycle is graphed over time for each of the clones.

The resistance to arrest cell cycle progression is a characteristic of cancer cells (1). One strategy for maintaining cell cycle progression despite lack of growth factors is to avoid cell cycle exit in to G0/quiescence. Therefore, we next wanted to determine whether the difference in response to growth factor removal by the T1 cells compared to the EL and empty vector cells was that the latter are entering a quiescent state where as the T1 cells do not, therefore ki67 staining of each clone was assessed during a 120hour period of growth factor deprivation. Ki67 is a nuclear protein involved in cell proliferation, although its role in proliferation is still unclear. It is expressed during G1, S and G2/M phases of the cell cycle and then is rapidly degraded. Therefore Ki67 is commonly used to differentiate cycling from non-cycling cells as it stains all phases of the cell cycle (i.e. G1, S and G2/M), but not G0. Nuclear staining with DAPI identified the Ki67 positive cells and then the individual cells were examined for Ki67 staining at 0, 24, 72 and 96 hours post growth factor deprivation (figure 2). At 0hrs the cells from all the clones are cycling through different phases of the cell cycle (except G0) asynchronously, as a result ~100% of the empty vector, EL and T1 overexpressing cells were ki67 positive. Because cells that have passed the restriction point must complete that round of the cell cycle, by 24 hours even if the cells had sensed growth factor deprivation, they were still in the cell cycle and therefore all clones (EL, T1 and empty vector) still had about 85% ki67 positive cells. Our previous data had shown that by 72 hours the entire population of empty vector expressing cells had arrested in G0 or G1 phase of the cell cycle. Furthermore, all these empty vector containing cells are ki67 negative, indicating that they are not cycling and are instead entering quiescence. Meanwhile, by 72 hours, approximately 30% of the EL overexpressing cells were KI67 positive, however the majority (~80%) of the T1 overexpressing cells were Ki67 positive. Subsequently, at 96 hours, the Ki67 staining of

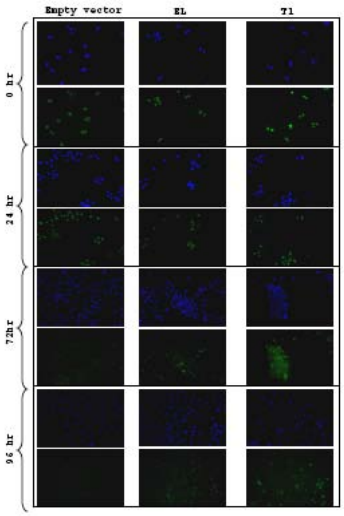

Figure 2: T1 overexpressing cells do not enter quiescence under growth factor deprived conditions. Each of the 76NE6 cell lines were collected at 0,24,72 and 96 hours after removal of growth factor deprivation and stained for DAPI(blue)and Ki67-FITC(green)

EL had diminished to virtually undetectable levels, where as there was still strong staining of Ki67 in the T1 overexpressing cells (figure 29). These data show that while the cells overexpressing full-length cyclin E or an empty vector control exit from the cell cycle and enter a quiescent state when challenged with growth factor deprivation, the T1 overexpressing cells do not arrest their cell cycle, resisting the normal regulation that would send the cell in to a quiescent state.

The insensitivity to anti-growth signals (growth factor deprivation), and resistance to quiescence are characteristic of a transforming phenotype- from normal to tumorigenic. Therefore, experiments were designed to serve as in vitro indicators of tumorigenicity to test whether the T1 overexpressing cells had a acquired a tumorigenic phenotype.

In our second aim we are examining the tumorigenic potential of non-tumorigenic cells overexpressing the cyclin E isoforms. The overexpression of LMW cyclin E results in deregulation of the cell cycle. In order to determine whether deregulation of the cell cycle by cyclin E affects the genomic fidelity of the cell, the 76NE6 clones overexpressing the cyclin E isoforms were arrested in metaphase and chromosomes were analyzed by karyotype analysis for gross chromosomal aberrations. Figure 3 shows representative karyotypes of metaphases from each of the 76NE6 clonal cell lines (Empty vector, EL and T1). The Empty vector karyotype was considered normal with all chromosomes being intact. However, chromosomal aberrations were identified in both the EL and T1 cell's karyotypes. Several of the chromosomal aberrations observed are shown alongside the karyotype in figure 3A. The structural aberrations that are visible in the metaphase spreads of these clones include: 1) dicentric chromosomes, chromosomes which contain two copies of the same centromere, 2) ring chromosomes, which results from 2 breaks that are attached to form a circular configuration, 3) breaks in one of the chromatids 4) fragments of a chromosome and 5) fusion of the telomeres of 2 chromosomes. This karyotype analysis was performed on 3 independent sets of the 76NE6-cyclin E clones. The results are shown in figure 3B. The percentage of chromosomal aberrations includes all metaphases that had at least one chromosome with any type of aberration. Statistical differences between the cell lines were determined using the student T-test with a 95% confidence interval. P-values less than 0.05 were considered

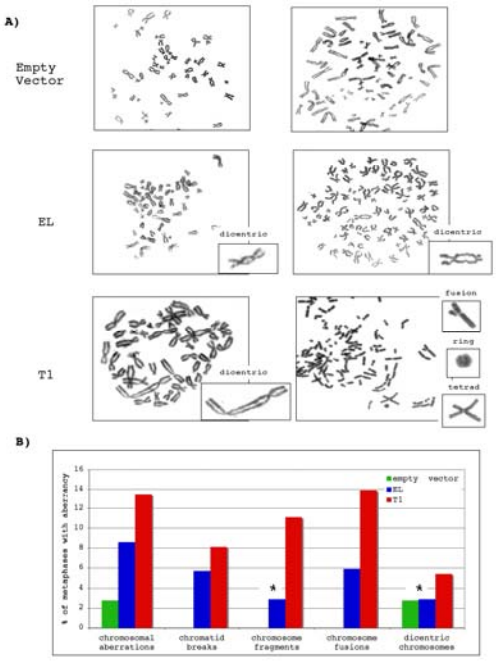

Figure 3: The T1 isoform of cyclin E generates genomic instability in 76NE6 cells. Metaphase spreads and karyotype analysis was performed on 76NE6 clones (empty vector, EL or T1 isoforms of cyclin E). A) karyotypes of the chromosomes from each clone. Examples of aberrant chromosomes within the metaphase are shown. B) Graph showing the percentage of metaphases analyzed with the given aberrations. For all types of aberrations assessed, the % of metaphases with that aberrancy was significantly differerent than the other 2 cell lines, except the 2 types of aberrancy marked with a * were not different between EL and empty vector cells

statistically significant. In each experiment, T1 overexpressing cells exhibited significantly more overall chromosomal abnormalities than the EL overexpressing cells. The empty vector expressing cells exhibited significantly less (or no) chromosomal aberrations. Chromatid breaks and chromosome fusions were found in significantly more metaphases of T1 overexpressing cells than EL or empty vector expressing cells. However the EL overexpressing cells had more metaphases with these aberrations than did the empty vector cells. The T1 cells also had more metaphases with chromosome fragments and dicentric chromosomes than the metaphases of EL or empty vector overexpressing cells. Furthermore, the EL cells were not statistically different from the empty vector cells in regards to the number of metaphases with chromosome fragments or dicentric chromosomes. Overall our results show that the full-length cyclin E does generate genomic instability in 76NE6 cells, however, T1 generates significantly more genomic instability than EL does. In all aberrations assessed, the T1 cells were more unstable than the empty vector cells, whereas the EL cells were not different from the empty vector cells in 2 out of the 5 types of chromosomal aberrations assesses. Therefore, 76NE6 cells become genetically unstable subsequent to LMW cyclin E expression.

Using the non-tumorigenic, 76NE6, cells stably expressing full-length cyclin E (EL), the LMW cyclin E (T1) or an empty vector (4.0), soft agar colony forming assays and matrigel invasion assays were performed. The conclusion was that cyclin E overexpression (full-length, or LMW isoform) does not provide the cells the ability to grow independently of cell adhesion. Despite negative results for the *in vitro* tests of tumorigenic potential using the 76NE6 cells overexpressing the cyclin E isoforms, we still wanted to test the ability of these clones to form tumors *in vivo*. An *in vivo* system is less stringent in terms of growth conditions compared to the *in vitro* system, i.e., *in vivo* the cells will have continuous access to growth factors and support of the extracellular matrix. Therefore, we rationalized that the agar based in vitro model systems may not be optimized for growing otherwise non-tumorigenic cells and used the mouse mammary fat pad as a host for growth of our 76NE6 cells overexpressing the cyclin E isoforms to test their tumorigenic potential.

The third aim of this proposal was to test the in vivo tumorigenic potential of LMW cyclin E (T1) in an otherwise non-tumorigenic cell line, injected the cells in to the mammary fat pad of nude mice. The mice were also irradiated to further weaken the immune system of the mouse to aid with implantation and growth of our human cell lines. 10 mice

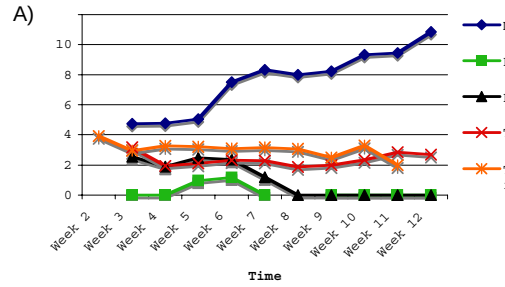

Figure 4: 76NE6 cells overexpressing the T1, but not EL, isoform of cyclin E form tumors in mice. $1X10^7$ 76NE6 cells overexpressing EL, T1 or the empty vector or MDA-MB-468 cells were injected in to 10 nude mice (T1 was injected in to 20 mice). A) The diameter of the tumor was measured and recorded weekly for each set of 10 mice. B) Staining of sections of the T1 tumor with hematoxylin and eosin (left) and cyclin E (right).

were injected with each of the 76NE6 clonal cell lines overexpressing empty vector or the full-length, EL, isoform of cyclin E. 20 mice were injected with the 76NE6 cells overexpressing T1 (10 were injected 24 hours post irradiation and 10 were injected 72 hours post irradiation). 10 mice were also injected with MDA-MB-468 cells that had been cultured from a tumor formed in mice from this cell line previously, as a positive control. Of each group of 10 mice, 5 were injected with cells suspended in matrigel and 5 were injected with cells suspended in media as we did not know whether these cells would need the support of the matrigel to keep them in close contact to initiate growth. Remarkable differences were observed between the incidence of tumor formation in the mice injected with the T1 isoform compared to the EL isoform of cyclin E. 3 months post injection, 100% of the mice harboring the T1 cells have evidence of tumor formation whereas none of the mice injected with the EL isoform have evidence of tumors figure 4A. Reassuring to the integrity of our model system was the fact that none of the mice injected with the 76NE6 cells expressing vector alone formed tumors. The tumors formed by the T1 cells are slow growing (figure 4A), after 3 months, the tumors average 2.7mm in diameter, whereas the tumors formed by the MDA-MB-468 cells reached 11cmm and 4 out of the 10 mice were sacrificed. The tumors from the T1 mice were stained with hematoxylin and eosin and were determined to be tumor tissue by a pathologist. The cyclin E expression was also confirmed by immunohistochemistry (figure 4B). From these data we can conclude that the T1 isoform of cyclin E, when overexpressed in a p53 negative, but otherwise non-tumorigenic epithelial cell line results in tumorigenesis.

Key Research Accomplishments:

The experiments performed in this study clearly show that the LMW isoforms of cyclin E play a direct and pivotal role in the tumorigenic process. This has been shown using a broad spectrum of assays and model systems, from in vitro mechanistic studies, to cell based assays and in vivo tumorigenicity assays. From these experiments, the following conclusions can be drawn:

(1) The low molecular weight (LMW) isoforms of cyclin E confer increased kinase activity to the CDK2/cyclin E complex
(2) Expression of the cyclin E isoforms along with CDK2 and the CKIs, p21 and p27, in sf9 cells showed that there are 3 main mechanisms that provide the LMW isoforms the ability to form a hyperactive complex with CDK2:

- The binding of the LMW isoforms of cyclin E to CDK2 is more efficient than the binding of full-length cyclin E to CDK2
- The LMW isoforms of cyclin E resist the inhibition of the CKIs, p21 and p27.
- p27 binds preferentially to the LMW cyclin E isoforms compared to the full-length cyclin E. Therefore, p27 is sequestered from the full length cyclin E. The preferential binding and resistance of the LMW cyclin E to p27 means that the overall activity of cyclin E/CDK2 is kept active despite CKI binding.

(3) The activity of the LMW cyclin E/CDK2 complex provides 76NE6 cells with a growth advantage as shown by the increased percentage of cells in S phase of the cell cycle and increased colony forming ability compared to full-length cyclin E.
(4) The LMW cyclin E makes 76NE6 unresponsive to lack of growth factors. Unlike the full-length cyclin E overexpressing cells, the LMW overexpressing cells do not enter quiescence when challenged with the lack of growth factors.
(5) The T1 isoform of cyclin E generates a more unstable genome in 76NE6 cells compared to the full-length cyclin E according to the number of metaphase cells with gross chromosomal aberrations observed in karyotypic analysis

(6) 76NE6 cells expressing the T1 isoforms can form tumors in nude mice at an incidence rate of 100%, whereas the cells expressing EL do not form tumors at all.

In conclusion, the LMW isoforms provide a novel mechanism of cell cycle deregulation in mammary epithelial cells that is involved in the tumorigenic process.

Reportable Outcomes

- Paper to be submitted, October 2007.
- Abstract submitted to AACR –Breast Cancer Research Meeting, October 2007.
- Work was presented and defended successfully as dissertation, May 2007.
- The cell lines generated in this proposal (76NE6/empty vector, 76NE6/EL and 76NE6/T1) have been given to other investigators to study cyclin E as a potential therapeutic target.

References:

1. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100(1):57-70.